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RESEARCH ARTICLE

α-Carbonic anhydrases are sulfatases with cyclic diol monosulfate esters

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Abstract

Carbonic anhydrases (CA) catalyze activated ester hydrolysis in addition to the hydration of CO_2 to bicarbonate. They also show phosphatase activity with 4-nitrophenyl phosphate as substrate but not sulfatase with the corresponding sulfate. Here we prove that the enzyme is catalyzing the synthesis of cyclic diols from sulfate esters. 5-, 6- and 8-membered ring cyclic sulfates incorporating a neighboring secondary alcohol moiety were treated with CA II and yielded the corresponding cyclic diols. Inhibitory properties of obtained cyclic and original sulfate esters were then investigated on human carbonic anhydrase I (hCA I), hCA II, hCA IV and hCA VI (h = human isoform). K_I-s of these compounds ranged between 32.7–423 μM against hCA I, 2.13–32.4 μM against hCA II, 13.7–234 μM against hCA IV and 76–278 μM against CA VI, respectively. The sulfatase activity of CA with such esters is amazing considering the fact that 4-nitrophenyl-sulfate is not a substrate of these enzymes.

Keywords: Cyclic diols, carbonic anhydrase, inhibition, antiglaucoma

Introduction

Mammals possess 16 different carbonic anhydrase (CA, EC 4.2.1.1) isoforms, which are involved in many crucial physiological processes connected with respiration and transport of CO_2/HCO_3^-, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions, bone resorption, etc[1–6]. Some of the isozymes are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), two are mitochondrial (CA VA and CA VB), one is secreted (CA VI), and others are membrane-bound (CA IV, CA IX, CA XII and CA XIV)[1–11]. In a recent preliminary work from our group, we investigated the interaction between natural phenolic compound, antioxidant phenolic compounds, hydroxy/metoxy organic compounds and salicylic acid derivatives with two cytosolic catalytically active isoforms (CA I and II) of the metalloenzyme CA[1–8]. Indeed, phenol 12 binds to CA in a diverse manner compared to the classical inhibitors of the sulfonamides/sulfamates/sulfamides, which coordinate to the Zn^{2+} ion from the enzyme active site by substituting the fourth, non-protein ligand, a water molecule or hydroxide ion[12–15]. Recently, Christianson's group then reported the X-ray crystal structure for the adduct of human carbonic anhydrase II (hCA II) with phenol[12], showing indeed this inhibitor to bind to hCA II by anchoring its OH moiety to the zinc-bound H_2O/hydroxide ion of the enzyme through a hydrogen bond as well as to the NH amide of Thr199, an amino acid conserved in all α-CAs and critically important for the catalytic cycle of these enzymes[4–6,12–15]. Furthermore, the phenyl moiety of this inhibitor was found to lay in the hydrophobic part of the hCA II active site, where presumably CO_2, the physiologic substrate of the CAs, binds in the precatalytic complex, explaining thus the behaviour of phenol as a unique CO_2 competitive inhibitor[1–3,13–16].

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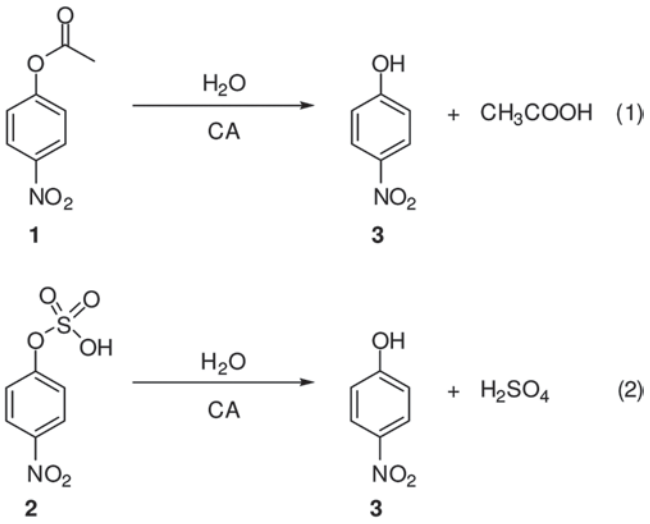
Inhibitory effects of different phenols, metoxyphenol derivatives, anions, metal ions and drugs have been investigated up to now against many mammalian, fish, bacterial and fungal CAs[12–20]. CA II is the physiologically most relevant isoenzyme. CA inhibitors (CAIs) are used for several applications, in particular for the treatment of glucoma, epilepsy, as diuretics etc. Other compounds, targeting isoforms IX and XII, have applications as antitumor agents/diagnostic tools[1–11]. Therefore, discovery of novel CAIs targeting various isoenzymes has gained attention nowadays[1–10]. Cyclic diols were developed treatments of type II diabetes, Gaucher disease and also as an anti-HIV drug[21–24]. In the current study, we aimed to synthesize cyclic diols 5, 7 and 9 using carbonic anhydrase enzyme and determine the inhibitory effects of cyclic diols and sulfate esters on four α-CA isozymes, hCA I, hCA II, hCA IV and hCA VI.

Results and discussion

Chemistry

It is known that carboxylate/phosphate esters are hydrolyzed by α-CAs, although 4-nitrophenylsulfate was shown not to be a substrate for the cytosolic isoforms hCA I, II and XIII[1–6]. Recently, one of our groups reported kinetic study on the hydrolysis of 4-nitrophenyl acetate 1 and phosphate in the presence of three cytosolic CA isozymes, hCA I, hCA II and hCA XIII[1–6]. In solution, these esters are hydrolyzed by the nucleophilic attack of water (or hydroxide ions) to the central atom (carbonyl CO for acetate 1, phosphorus for phosphate) with formation of a transition state from which the 4-nitrophenoxide is released. Considering the fact that CAs contain the equivalent of a strong base (hydroxide ions, HO^- coordinated to the zinc ion) at neutral pH, due to the powerful activation of H_2O by the zinc ion from the active site cavity and the hydrophobic environment of the protein, in principle, hydrolytic reactions 1–2 of Scheme 1 should have the same mechanism as the hydrolysis catalyzed by bases in solution. In these studies, Innocenti et al.[1,2] showed that the hydrolytic processes described by Eqs. 1–2 of Scheme 1, involve the active site $Zn^{2+}(OH)^-$ functionality of the enzyme, that is, the same one responsible of the CO_2 hydration activity of α-CAs. Probably, compounds 4, 6 and 8 are hydrolysed by CA II in the same way in the current study. It is interesting to note here that the aliphatic, cyclic sulfates investigated here, unlike the aromatic activated one (ester 2) indeed act as substrates for CAs. The sulfatase activity of this enzyme has been in fact discovered earlier with a cyclic sulfate ester as substrate, by Kaiser and Lo[3].



Scheme 1. Reactions 1 and 2 catalyzed by α-carbonic anhydrases (CAs). Whereas the 4-nitrophenyl acetate hydrolysis occurs easily, the corresponding sulfate 2 is not a substrate for CAs[1–3].

Furthermore, we report here an inhibition study of the four catalytically active human isoforms hCA I, II, IV and VI with compounds 4–11. They incorporate sulfate esters or cyclis diols in their molecules and scaffolds representing thus an interesting starting point for different chemotypes belonging to the CAIs. In fact, in an earlier study[25] we reported micromolar/submicromolar inhibitors of the cytosolic isoforms hCA I and II with a library of organic nitrates.

CA purification, assay and inhibition

The purification of hCA isozymes was performed with a simple one step method by a Sepharose-4B-aniline-sulfanilamide affinity column[9–11]. Inhibitory effects of compounds trans-(1*R*(*S*),6*R*(*S*))-6-Hydroxycyclohex-3-enyl hydrogen sulfate (4), (1R,2R)-cyclohexane-1,2-diol (5), trans-(*1R*(*S*),8*R*(S),*Z*)-*8*-Hydroxycyclooct-4-enyl hydrogen sulfate (6), (2*R*,3*R*)-1,2,3,4-tetrahydronaphthalene-2,3-diol (7), 9(*R*(*S*))-Hydroxy-1,2,3,4-tetrahydro-1,4-methanonaphthalen-2(*R*(*S*))-yl sulfate (8), 9(*R*(*S*))-Hydroxy-1,2,3,4-tetrahydro-1,4-methanonaphthalen-2(*R*(*S*))-diol (9), trans-(1*R*(*S*),2*R*(*S*))-cyclohexane-1,2-diol (10) and trans-(2*R*(*S*),3*R*(*S*))-1,2,3,4-Tetrahydronaphthalene-2,3-diol (11) on these isoenzyme activities were tested under *in vitro* conditions; K_I values were calculated from Lineweaver-Burk plots and are given in Table 1[25–29].

The CA isozymes play important roles in different tissues[1–9,28–32]. It is known that CA has been purified many times from different organisms and the effects of various chemicals, pesticides, anions, metal ions and drugs have been investigated on its activity[8–20,28–32]. We report here a study on the inhibitory effects of organic sulfates, diols and some phenolic compounds on the CA esterase activity of isoforms hCA I, II, IV and VI. Data of Table 1 show the following, regarding inhibition of hCA I, II, IV and VI with compounds 4–11 and with positive controls 12, 13, 14, Acetazolamide (AZA):

1. Against the slow cytosolic isozyme hCA I, compounds 5, 7, 9–10 behave as weak, micromolar inhibitors, with K_I values the range of 135–188 μM. Compound 11 was an ineffective hCA I inhibitor (K_I of 423 μM). A second group of derivative, including 4, 6 and 8, showed better inhibitory activity as compared to the previously mentioned compounds 5 and 7, with K_I values of 32.7–77.2 μM, (Table 1). Compound 11 showed K_I value of 423 μM, and various substutions

Table 1. K_I values obtained from regression analysis graphs for hCA I, hCA II, hCA IV, and hCA VI in the presence of different inhibitors concentrations (μM).

Inhibitor	hCA-I	hCA-II	hCA-IV	hCA-VI
4	41.3	2.13	13.7	93.1
5	163	4.08	23.6	116
6	77.2	5.41	16.3	123
7	188	8.27	53.8	145
8	32.7	10.4	199	221
9	142	23.7	234	278
10	135	4.68	18.8	76.2
11	423	32.4	77.9	127
Phenol (12)	10.2*	5.5*	9.5*	208*
Catechol (13)	4003*	9.9*	10.9*	606*
Resorcinol (14)	795*	7.7*	570*	550*
Acetazolamide	36.2	0.37	5.64	0.34

Mean from at least three determinations. Errors in the range of ±3% of the reported value (data not shown).
hCA, human carbonic anhydrase.
*From reference 13.

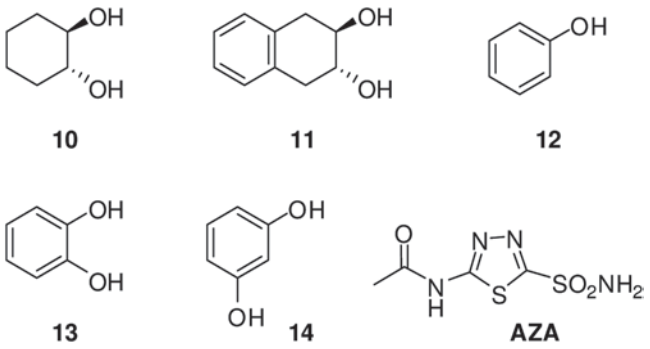
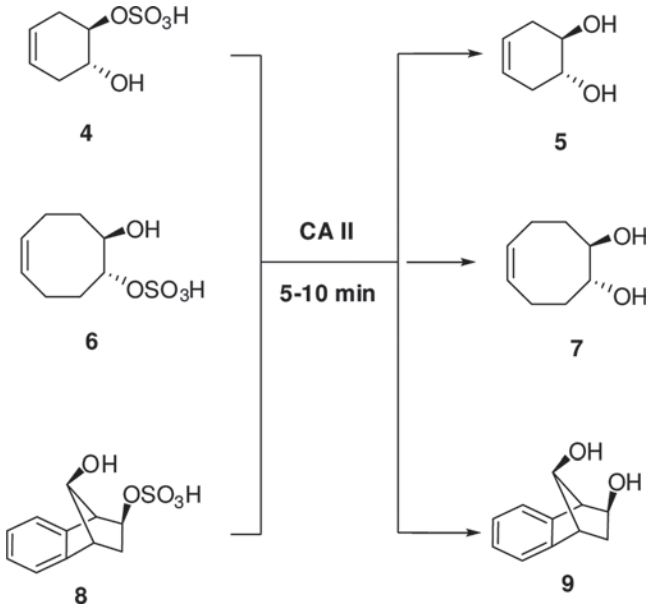


Figure 1. Structure of compounds 10–14 and (AZA).

patterns such as the introduction of the hydroxyl and sulfate- moieties lead to minor changes in activity, compounds 5, 7, 9–11 behaving as rather weak hCA I inhibitors (Figure 1). The same effect is observed when the inhibition constants are calculated (Table 1) by means of Lineweaver-Burk plots, these compounds showing K_I-s in the range of 135–423 μM.

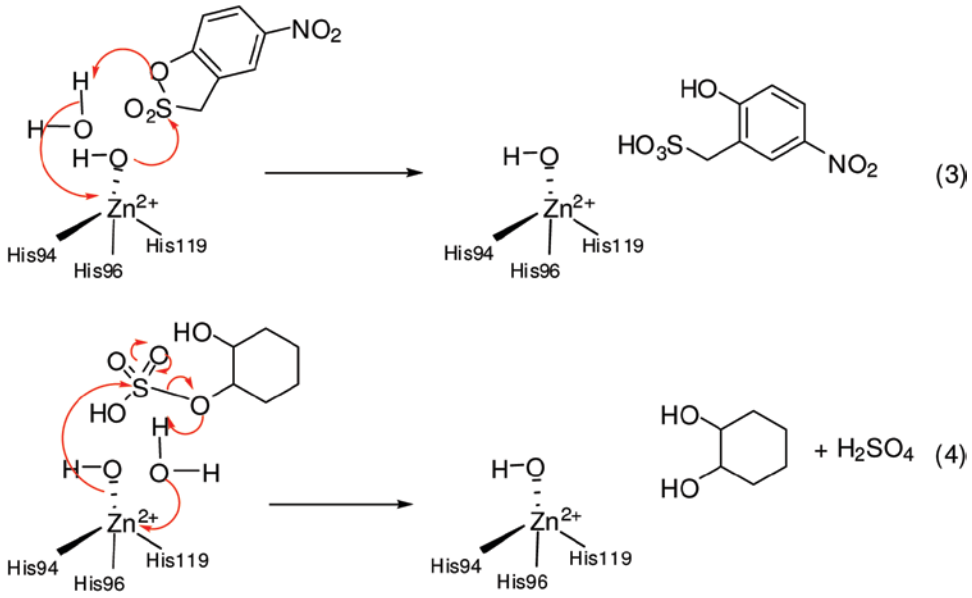
These compounds incorporate moieties leading to an acidification of the OH groups form the organic sulfates and cyclic diols scaffold (such as OSO_3H or OH group in the 2-or 3-position in compound 4 and 5), as well as the bulkier scaffolds present in 6, 7 and especially 8, 9, 11. These were among the best inhibitors in this series of organic sulfates and cyclic diols. Data of Table 1 also show that similarly to phenolic compounds[9–15], most of the investigated organic sulfates (4, 6 and 8), act as competitive inhibitors with 4-NPA as substrate, i.e. they bind in the same regions of the active site cavity as the substrate. However, the binding site of 4-nitrophenylacetate (NPA) itself is unknown, but it is presumed to be in the same region as that of CO_2, the physiological substrate of this enzyme[12–15]. Similarly to salicylic acid derivatives and phenolic compounds investigated earlier by us, the investigated compounds



Scheme 2. The hydrolysis reaction of compound 4, 6 and 8 with carbonic anhydrase II (CA II) isoenzyme.

act as competitive inhibitors with 4-NPA as substrate, that is, they bind in same regions of the active site cavity as compared to the substrate.

2. A better inhibitory activity has been observed with compounds 4–6, 10 investigated here for the inhibition of the rapid cytosolic isozyme hCA II (Table 1). Four derivatives, i.e. 7–9, 11 showed moderate hCA II inhibitory activity with K_I-s in the range of 10.4–32.4 μM (Table 1), whereas the remaining four derivatives were quite effective hCA II inhibitors, with K_I-s in the range of 2.13–5.41 μM (Table 1). Structure-activity relationship (SAR) is thus quite sharp for this small series of tetralin scaffold compounds (8, 9 and 11) are ineffective leads. The best hCA II inhibitor in this series of derivatives 4.
3. Compound 11, and some of its congeners such as compounds 8 and 9 are also weak inhibitors of CA IV, with K_I-s of 77.9–234 μM. However, again compound 7 is medium potency inhibitor (K_I of 53.8 μM), and compounds 4–6 and 10 show a higher affinity for this isozyme, with inhibition constant in the range of 13.7–23.6 μM, AZA with K_I of 5.64 μM (Table 1).
4. Phenol 12 and some of its congeners such as 13 and 14 are also weak inhibitors of the secreted isozyme hCA VI, with K_I-s of 208–550 μM[13]. However, again the compounds 8 and 9 are medium potency inhibitors (K_I of 221–278 μM), and derivatives 4–7, 10 and 11 show higher affinity for this isozyme, with inhibition constants in the range of 76.2–145 μM (Table 1).
5. We hypothesize that CAs (which as we show above, possess esterase activity against several substrates), hydrolyses these organic sulfates leading to sulfuric acid and cyclic diols, as illustrated in Scheme 2. Previously, studies showed the hydrolysis reaction of 2-Hydroxy-5-nitro-α-toluenesuIfonic acid sultone (Scheme 3)[1–3].



Scheme 3. The hydrolysis reaction of 2-Hydroxy-5-nitro-α-toluenesulfonic acid sultone[3] (3) and cyclic sulfate esters (4).

In recent studies, it was reported that phenols and natural phenolic compounds act as CAIs, and could represent the starting point for a new class of inhibitors that may have advantages for patients with sulfonamide allergies (thioxolone acts as a prodrug)[8–16].

Cyclic diols or cyclitols are receiving considerable attention as chemotherapeutic agents against diabetes, cancer, and viral infections[26–29]. Several multiple sulfated compounds have been found in biologically active compounds and marine organisms[28]. For instance, sulfated sterols have exhibited effects such as anti-HIV, antiviral activity, and inhibition of protein tyrosine kinases[26–28]. Recent studies also showed that sulfo-containing salicylic acid derivatives have inhibitory effects on CA I and II isoenzymes[9]. However, it is critically important to explore further classes of potent CAIs in order to detect compounds with a different inhibition profile as compared to the sulfonamides and their bioisosteres and to find novel applications for the inhibitors of these widespread enzymes, in particular tumor-related isoforms.

Conclusions

Cyclic diols 5, 7 and 9 were synthesized from sulfate esters 4, 6 and 8 using CA II isoenzyme. Cyclic diols and sulfate esters 4–11 affect the activity of CA isozymes due to the presence of the functional group (OH) in their cyclic aliphatic scaffold. Our findings indicate thus another class of possible CAIs of interest, in addition to the well-known sulfonamides/sulfamates/sulfamides, although their mechanism of CA inhibition remains rather elusive at this moment. We have reported several inhibitors of both CA and other proteins so far[30–44] and discovery of novel inhibitors still requires further investigations. Indeed, some cyclic diols investigated here showed effective CA I, II, IV and VI inhibitory activity, in the micromolar range, by the esterase assay method. These findings point out that substituted cyclic diols may be used as leads for generating more potent CAIs eventually targeting other isoforms which have not been assayed yet for their interactions with such agents.

Experimental

Chemicals

Sepharose 4B, protein assay reagents, *p*-nitrophenol, 4-nitrophenylacetate and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. All other chemicals were of analytical grade and obtained from Merck.

Purification of CA isozymes from human blood by affinity chromatography

Fresh citrated human whole blood obtained from the Blood Center of the Research Hospital at Atatürk University. Cells were washed three times by centrifugation at 1000xg at 4 ± 6°C, for 20 min in four volumes of 25 mM Na_2HPO_4 (pH 7,4) buffer. Supernatant and fluffy coat were removed. The erythrocytes were lysed in 10 volumes of 5 mM Na_2HPO_4 (pH 7.4) buffer, containing 1 mM EDTA. After 20 min, the haemolysate was centrifuged at 10,000*g* for 60 min. The particulate fraction was washed four times in the same buffer. The membranes were centrifuged down at 15,000*g* for 60 min. pH was adjusted to 8.3 with solid Tris. Sepharose-4B-aniline-sulfanilamide affinity column equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.3). The affinity gel was washed with 25 mM Tris-HCl/25 mM Na_2PO_4 (pH 8.3). Finally, human carbonic anhydrase IV (hCA IV) isozyme was eluted with 25 mM Tris-HCl/0.5 M $NaClO_4$ (pH 7.4)[45,46]. Fresh non-citrated human whole blood obtained from the Blood Center of the Research Hospital at Atatürk University. The

Table 2. Time-% conversion rate with buffer or H_2O medium (rt, 1 atm).

	Conversion rate							
Compound	15 min	30 min	45 min	60 min	90 min	120 min	150 min	180 min
4	0.5%	7%	35%	53%	67%	93%	97%	>99%
6	0	1%	11%	25%	34%	51%	73%	86%
8	0	0	0	0	0	<1%	1%	1%

blood samples were centrifuged at 5000 rpm for 15 min and precipitant were removed. The serum was isolated. The pH was adjusted to 8.7 with solid Tris. Sepharose-4B-aniline-sulfanilamide affinity column equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25mM Tris-HCl/22mM Na_2SO_4 (pH 8.7). The hCA-VI isozyme was eluted with 0.25 M H_2NSO_3H/25 mM Na_2HPO_4 (pH 6.7). All procedures were performed at 4°C[16,47].

Enzyme mediated synthesis of cyclic diols

The reactions were performed in the presence of hCA II in water at pH 7.5. A 10-fold excess of the starting sulphate esters 4, 6, 8 was used to limit side reactions. Three reactions were performed with and without enzyme in a sodium phosphate solution at pH 7.4 (20 mM phosphate buffer). Stock solution in dimethyl sulfoxide of three sulphate esters (10 mM) were added to three aqueous solutions in order to reach the final concentration of 0.08 mM. The clear mixture was incubated at 25°C for 5 min, for 4 and 6 and for 10 min, for 8. The sulphate esters yielded the corresponding diols in 100% yield in 5–10 min although 4 and 6 hydrolyzed without enzyme solution in approximately 150 min whereas 8 was not hydrolyzed without the enzyme (Table 2). Prior to analyzing the products, the mixture was left for 2 h to be separated from CA by decantation. The thermal denaturation of the enzyme (2 min at 80°C) was also tested to ensure the release from casting site of some possible tightly bound ligands.

CA inhibition

CA activity was assayed by following the change in absorbance at 348 nm of 4-NPA to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer (Shimadzu UV-VIS) according to the method described by Verpoorte et al.[48] The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05M Tris-SO_4 buffer (pH 7.4), 1 mL, 3 mM NPA, 0.5 mL H_2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. The inhibitory effects of compounds 4–11 were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor an Activity%- [Inhibitor] graph was drawn. To determine K_I values, three different inhibitor concentrations were tested. In these experiments, NPA was used as substrate at five different concentrations (0.15–0.75 mM). The Lineweaver-Burk curves were drawn[49].

Protein determination

Protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard[50].

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the enzymes. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli[51].

Synthesis of sulfate esters

Detailed synthetic procedures for the preparation of all derivatives can be found in: Ref 37.

Declaration of interest

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